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High Prevalence of GB Virus C Strains Genetically Related to Strains With Asian Origin in Nicaraguan Hemophiliacs

Mario A. González-Pérez,[1,3] Heléne Norder,[1]* Åsa Bergström,[1] Ernesto Lopéz,[2] Kirsten A. Visoná,[3] and Lars O. Magnius[1]

[1]*Department of Virology, Swedish Institute for Infectious Disease Control, Stockholm, Sweden*
[2]*Red Cross Blood Program, Red Cross of Nicaragua, Managua, Nicaragua*
[3]*Louisiana State University–International Center for Medical Research and Training, San José, Costa Rica*

The presence of hepatitis GB virus C (GBV-C), also known as hepatitis G virus (HGV), and hepatitis C virus (HCV) were investigated in sera from 45 hemophiliacs from nine locations in Nicaragua using a nested polymerase chain reaction (PCR). Primers used to detect GBV-C and HCV derived from the helicase region and 5′UTR, respectively. Seventeen (38%) patients were positive for GBV-C RNA in serum by PCR. Twelve (27%) patients were positive for HCV RNA by PCR. Six (13%) of these were coinfected with GBV-C. Anti-HCV was detected in all the 12 HCV RNA positive hemophiliacs and in another 14 (31%) individuals, in whom GBV-C RNA was found in 2. Ten patients (22%) lacked markers for both GBV-C and HCV. The mean age of the patients positive for GBV-C but negative for HCV by PCR was significantly lower than for those negative for GBV-C but positive for HCV by PCR ($P < 0.05$; Student's t-test), indicating that the risk for this group of hemophiliacs to acquire GBV-C infection is higher as compared to the risk of acquiring HCV infection. Eleven GBV-C strains were sequenced in the 5′UTR. Sequence comparison to previously published GBV-C strains revealed that all 11 strains were more similar to Asian strains than to strains of European and African origin. Sequences in the NS5-B region were available for 8 HCV strains, all of which were found to belong to genotype 1a. The similarity of the Nicaraguan GBV-C strains to strains from Asia indicates that the GBV-C strains in the region presumably have an Amerindian origin. It is also considered that the HTLV II strains in the New World aboriginal populations are ancient and brought there by the ancestral Amerindian populations from Asia. Further, the genotype F of hepatitis B virus, known to represent the strains in populations with Amerindian background, predominates in Central American populations with Hispanic background. It remains to be clarified why Amerindian strains of GBV-C as well as of HBV predominate also in populations with mixed ethnic background in Central America. ***J. Med. Virol. 52:149–155, 1997.*** © Wiley-Liss, Inc.

KEY WORDS: GBV-C; sequences; hemophiliacs; Amerindians; Central America

INTRODUCTION

A new virus designated GBV-C implicated in the etiology of viral hepatitis was described recently [Simons et al., 1995]. This virus is a positive single-stranded RNA virus of the Flaviviridae family more related to the previously discovered GBV-A and GBV-B and hepatitis C virus (HCV) than to pestiviruses and true flaviviruses, e.g., yellow fever virus, dengue viruses, and others [Simons et al., 1995]. Another recently described virus designated as hepatitis G virus (HGV) [Linnen et al., 1996] shares 95% homology at the amino acid level with GBV-C and therefore just represents another isolate of this virus [Zuckerman, 1996]. In contrast to HCV, for which 11 different genotypes are described with up to 30% variation based on the complete open reading frame [Choo et al., 1991; Okamoto et al., 1992; Tokita et al., 1994a; 1994b; 1995], GBV-C is a highly conserved virus with a divergency at the amino acid level of the polyprotein of only 4% between strains from the United States and Europe represented by HGV and African strains represented by GBV-C. However, based on phylogenetic analysis of the 5′-UTR,

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*Correspondence to: Heléne Norder, Virological Department, Swedish Institute for Infectious Disease Control, S105 21 Stockholm, Sweden.

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there are 3 different GBV-C clusters representing strains of varying geographical origin, tentatively designated as genotypes 1, 2, and 3 [Muerhoff et al., 1996].

The presence of divergent genomes of several different hepatitis viruses, e.g., hepatitis B virus [Naumann et al., 1993], hepatitis E virus [Huang et al., 1992], and hepatitis D virus [Casey et al., 1993], confined to certain ethnic groups in America, directs the attention of investigators to search for divergent viral genotypes in the area. So far, only scant information is available regarding HCV genotypes in Central America [Yun et al., 1996] and no information is available regarding the genetic diversity of GBV-C. This study was conducted in order to determine the presence of GBV-C and the genotypes of HCV in a group of hemophiliac patients at high risk of contracting viral hepatitis in Nicaragua.

MATERIALS AND METHODS

Study group

Serum samples from 45 hemophiliac patients (age range: 1–31 years, mean ± SD: 12.5 ± 7.1) from nine different locations in Nicaragua, were obtained, aliquotted, and shipped on dry ice to the Department of Virology at the Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

Serological Methods

Sera were tested for the presence of anti-HCV using a second generation anti-HCV test (Abbott Laboratories, Chicago, IL) at Red Cross Blood Bank, Managua, Nicaragua. Positive samples were analysed with the recombinant immunoblot assay, RIBA HCV 3.0 (Chiron, Emeryville, CA) at the Swedish Institute for Infectious Disease Control.

RNA Extraction and cDNA Synthesis

RNA was extracted from serum according to Garson et al. [1990] with minor modifications. Briefly, 100 μl serum was precipitated with 10% polyethylene glycol in 0.03M NaCl for 40 minutes on ice, the tubes were centrifuged, and the precipitate was dissolved in 125 μl of a buffer containing 5mM NaCl, 10mM Tris-HCl pH 8.3, 1mM EDTA, pH 7.0, and 25ng tRNA; 40 μl of a second buffer consisting of 5% sodium dodecyl sulphate, 25mM EDTA pH 7.0, and 250mM potassium acetate was added and the tubes were shaken vigorously at room temperature for 5 minutes. After phenol and phenol/chloroform extraction, the nucleic acids were ethanol precipitated. The RNA was dissolved in 20 μl diethyl-pyrocarbonate treated water and 5 μl dissolved RNA was used for cDNA synthesis using 1 U Superscript II reverse transcriptase (Gibco, BRL) and 1 nmole of random hexamer primers (Boehringer Mannheim).

GBV-C PCR and Sequencing

The NS3 region of the cDNA was amplified by PCR using primers GB1 (5′-GCCAACCCKAGGCAGATGCT-3′) at position 4035–4054 of the GBV-C genome according to Simons et al. [1995] and GB2 (5′-GAYAGCGCGTCTGTMGCACA-3′) at position 4433–4452. The product obtained was nested with primers GBVC-s1/GBVC-a1 according to Leary et al. [1996]. The 5′-UTR was amplified by PCR using primers S1/GBVCE1wb2 according to Muerhoff et al. [1996]. Five μl of the product was further amplified with the primers S1/4R [Muerhoff et al., 1996]. The PCR product was purified with Wizard PCR preps DNA purification system (Promega) and 0.5 pmoles were sequenced according to Casanova et al. [1990] in both directions with 5 pmoles of the primers S1 and 4R.

HCV PCR and Sequencing

HCV RNA was detected in the samples by amplification of the cDNA in the 5′-UTR with the primer pair NCR1/NCR2 [Garson et al., 1990]. Five μl of the obtained product was further amplified with primers NCR3/NCR4 [Garson et al., 1990].

The NS5-B region of the cDNA was amplified by PCR using primers hep120 (5′-TGCGCGACBGABACRTTKGAGGA-3′) at position 8702–8724 of the HCV genome according to Choo et al. [1991] and primer hep101 (5′-ATACCCGCTGCTTTGACTC-3′) at position 8258–8276. The product obtained was amplified further with hep101b as hep 101 with 5′-biotinylation and hep 105 (5′-ATACCTAGTCATAGCCTCCGTGA-3′) at position 8616–8638. The PCR product was purified with Wizard PCR preps DNA purification system (Promega) and used for solid-phase DNA sequencing. One pmol of the purified product was immobilized on magnetic beads (Dynabeads M280-strepavidin; Dynal AS) and subsequently denatured to obtain single stranded DNA [Leitner et al., 1995]. The sequencing reaction was carried out for both strands using the reagents provided in the Sequenase Version 2.0 DNA Sequencing Kit (USB Cleveland, OH), [^{35}S]dATP, 2 units of Sequenase (USB), and 5 pmoles of the primers used in the PCR as sequencing primers. The sequenced fragments were electrophoresed on 6% polyacrylamide gels and autoradiographed.

Tree Constructions

Of the sequences obtained, 375 bp were aligned with the corresponding part of the genome of 49 GBV-C sequences available in EMBL GeneBank (Table I) and with GBV-C sequences in the sera from three Swedish drug addicts. Dendrograms were created with the programs DNADIST together with NEIGHBOUR (UPGMA) in the PHYLIP package, version 3.53 [Felsenstein, 1993]. SEQBOOT was used to bootstrap data, in which 100 data sets were analysed, and CONSENSE was used to compute consensus trees. For visualization and graphical editing of the trees, the program TREETOOL, version 1.0, in GDE was used.

RESULTS

Results are summarized in Tables II and III. GBV-C RNA was detected by nested-PCR using primers in the NS3 region in seventeen (38%) of the hemophiliacs (age range: 2–21; mean ± SD: 12.3 ± 6.4). Twenty-eight

TABLE I. Designation and GenBank Accession Numbers of the GBV-C Sequences Used in the Comparison

Designation	Genotype	Origin	Accession no.	Reference
Isolate 23	1a	West Africa	U59540	Muerhoff et al., 1996
Isolate 24	1a	West Africa	U59541	Muerhoff et al., 1996
Isolate 25	1a	West Africa	U59542	Muerhoff et al., 1996
Isolate 26	1a	West Africa	U59543	Muerhoff et al., 1996
Isolate 27	1a	West Africa	U59544	Muerhoff et al., 1996
Isolate 28	1a	West Africa	U59545	Muerhoff et al., 1996
Isolate 29	1a	West Africa	U59546	Muerhoff et al., 1996
GBV-C	1b	West Africa	U36330	Leary et al., 1996
Isolate 31	1b	West Africa	U59547	Muerhoff et al., 1996
Isolate 32	1b	West Africa	U59548	Muerhoff et al., 1996
Isolate 33	1b	West Africa	U59549	Muerhoff et al., 1996
Isolate 34	1b	West Africa	U59550	Muerhoff et al., 1996
Isolate 35	1b	West Africa	U59551	Muerhoff et al., 1996
Isolate 36	1b	West Africa	U59552	Muerhoff et al., 1996
Isolate 37	1b	West Africa	U59553	Muerhoff et al., 1996
Isolate 38	1b	West Africa	U59554	Muerhoff et al., 1996
Isolate 39	1b	West Africa	U59555	Muerhoff et al., 1996
Isolate 40	1b	West Africa	U59556	Muerhoff et al., 1996
Isolate 41	1b	West Africa	U59557	Muerhoff et al., 1996
Isolate 42	1b	West Africa	U59558	Muerhoff et al., 1996
PNF2161	2a	USA	U44402	Linnen et al., 1996
Isolate 1	2a	USA	U59518	Muerhoff et al., 1996
Isolate 2	2a	USA	U59519	Muerhoff et al., 1996
Isolate 3	2a	Europe	U59520	Muerhoff et al., 1996
Isolate 4	2a	Europe	U59521	Muerhoff et al., 1996
Isolate 5	2a	Europe	U59522	Muerhoff et al., 1996
Isolate 6	2a	USA	U59523	Muerhoff et al., 1996
Isolate 7	2a	USA	U59524	Muerhoff et al., 1996
Isolate 8	2a	USA	U59525	Muerhoff et al., 1996
Isolate 9	2a	USA	U59526	Muerhoff et al., 1996
Isolate 10	2a	Europe	U59527	Muerhoff et al., 1996
Isolate 11	2a	Europe	U59528	Muerhoff et al., 1996
Isolate 12	2b	Europe	U59529	Muerhoff et al., 1996
Isolate 13	2b	Europe	U59530	Muerhoff et al., 1996
Isolate 14	2b	USA	U59531	Muerhoff et al., 1996
Isolate 15	2b	USA	U59532	Muerhoff et al., 1996
Isolate 16	2b	Europe	U59533	Muerhoff et al., 1996
Isolate 17	2b	Europe	U59534	Muerhoff et al., 1996
Isolate 18	2b	USA	U59535	Muerhoff et al., 1996
Isolate 19	2b	USA	U59536	Muerhoff et al., 1996
Isolate 20	2b	Europe	U59537	Muerhoff et al., 1996
Isolate 21	3	Asia	U59538	Muerhoff et al., 1996
Isolate 22	3	Asia	U59539	Muerhoff et al., 1996
K606	3	Japan	D87249	Fukushi et al., 1996, unpublished
K1737	3	Japan	D87250	Fukushi et al., 1996, unpublished
K1789	3	Japan	D87251	Fukushi et al., 1996, unpublished
K1916	3	Japan	D87252	Fukushi et al., 1996, unpublished
K2141	3	Japan	D87253	Fukushi et al., 1996, unpublished
K2142	3	Japan	D87254	Fukushi et al., 1996, unpublished

(62%) of the hemophiliacs were GBV-C RNA negative in serum by PCR (age range: 1–31; mean ± SD: 12.7 ± 7.6). HCV RNA was detected by nested-PCR using primers from the 5′UTR in 12 (27%) of the hemophiliacs (age range: 8–31; mean ± SD: 16.5 ± 6.2). Twenty-six (58%) of the hemophiliacs were anti-HCV positive (age range: 5 to 31; mean ± SD: 14.4 ± 6.1), while 19 (42%) were anti-HCV negative by EIA (age range: 1–23; mean ± SD: 10.2 ± 7.8). All the anti-HCV positive but HCV RNA negative samples were also tested by RIBA and confirmed positive. Dual infections with GBV-C and HCV were detected in 8 (18%) of the patients (age range: 8–20; mean ± SD: 14.4 ± 4.5), of whom 2 were anti-HCV positive but negative for HCV RNA. Ten (22%) of the hemophiliacs were negative for both viruses (age range: 1–23; mean ± SD; 9.6 ± 8.5). HCV RNA was more often detected in sera of hemophiliacs above 10 years in age than in the younger age

TABLE II. Frequencies of HCV and GBV-C Markers in 45 Nicaraguan Hemophiliacs

Age range (yrs)	n	GBV-C RNA	HCV RNA	Anti-HCV	Any marker
1–10	19	7 (37%)	2 (10%)	8 (42%)	13 (68%)
11–20	19	9 (47%)	8 (42%)	14 (74%)	17 (89%)
21–>30	7	1 (14%)	2 (29%)	4 (57%)	5 (71%)
Total	45	17 (38%)	12 (27%)	26 (58%)	35 (78%)

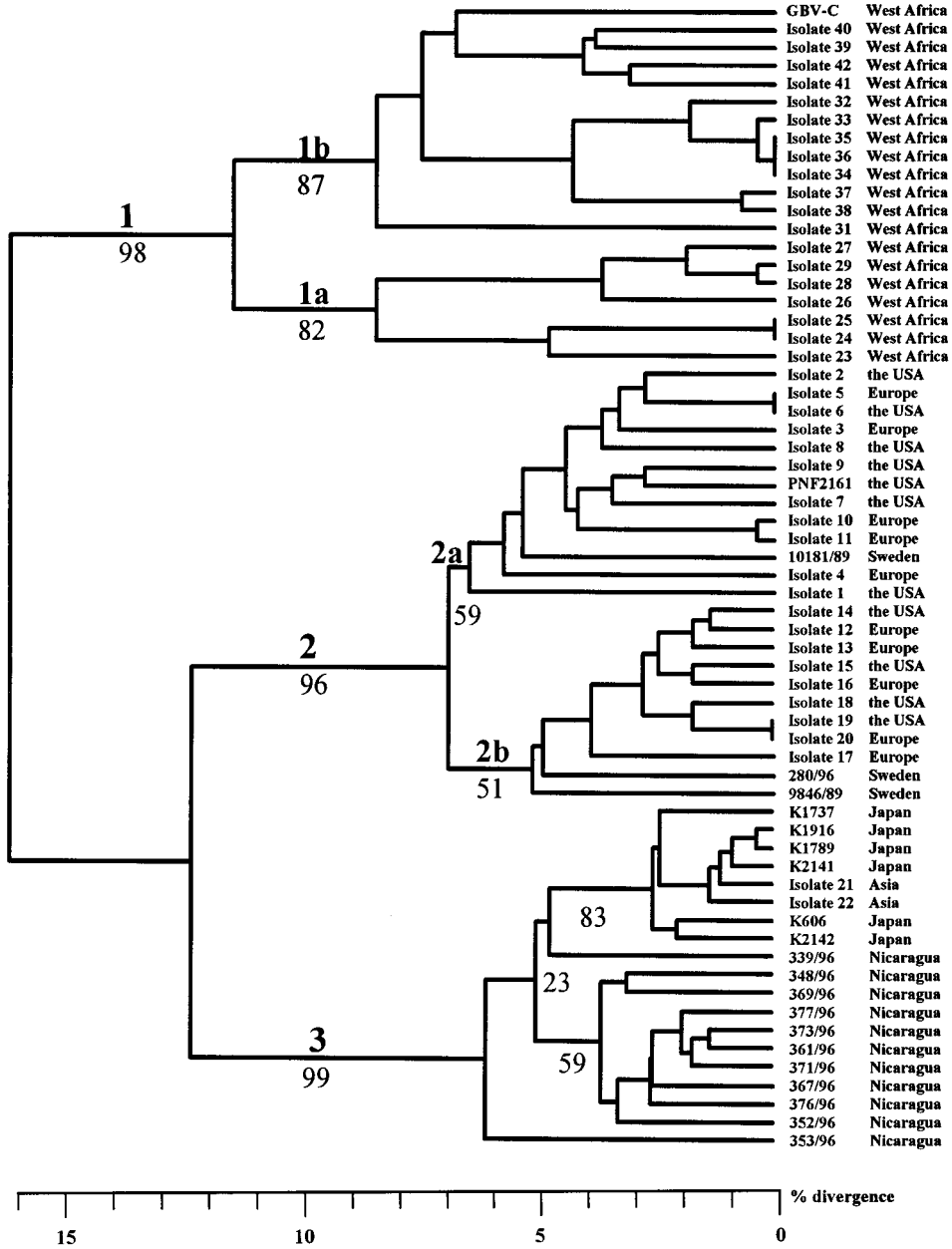

Fig. 1. Dendrogram obtained with UPGMA based on the 5′UTR sequences from 11 Nicaraguan hemophiliacs and 49 GBV-C sequences retrieved from GeneBank and 3 sequences from GBV-C strains from Swedish drug addicts. The figures at the forks of the dendrogram indicate the bootstrap values obtained from 100 replicas.

group, 10 out of 26 versus 2 out of 19 ($P < 0.05$; Fisher's test). No such relation was found for the presence of GBV-C RNA or anti-HCV. The mean age of patients positive for GBV-C but negative for HCV by PCR was significantly lower than for those negative for GBV-C but positive for HCV by PCR, 11.0 versus 18.3 years, respectively ($P < 0.05$; Student's t-test). Thus GBV-C infections in the Nicaraguan hemophiliacs seem to occur earlier than the infections with HCV.

As shown in Figure 1, a phylogenetic analysis of 5′UTR in 11 of the 17 Nicaraguan GBV-C strains showed that they were most similar to Asian GBV-C strains, although they formed a separate cluster as compared to the Asian strains provisionally designated as genotype 3 [Muerhoff et al., 1996].

Sequences from the NS5-B region were available in 8 of the 12 HCV strains recovered, all of which were found to belong to genotype 1a.

DISCUSSION

High prevalence rates for both GBV-C and HCV were observed in the group of hemophiliacs studied and in all age groups in agreement with previous studies reporting a high risk for HCV infection [Brettler et al., 1990; Rumi et al., 1990; Makris et al., 1990; Simmonds

Table III. Age Distribution of Nicaraguan Hemophiliacs According to Detection of GBV-C and HCV RNA by PCR

	GBV-C and HCV RNA			
Characteristic	GBV-C (+) HCV (–)	GBV-C (+) HCV (+)	GBV-C (–) HCV (+)	GBV-C (–) HCV (–)
Number of cases	11	6	6	22
Mean age ± SD	11.0 ± 6.8[a]	14.7 ± 5.3	18.3 ± 7.0[a]	11.1 ± 7.1
Anti-HCV	2 (18%)	6 (100%)	6 (100%)	12 (54%)

[a]$P < 0.05$; Student's t-test.

et al., 1990; Naoumov and Rumi, 1991] as well as an increased risk for GBV-C infection in patients with this background [Schleicher et al., 1996; Tagariello et al., 1996]. Dual infections have also been reported owing to both viruses sharing transmission pathways [Masuko et al., 1996; Nübling and Lower, 1996; Feucht et al., 1996; Linnen et al., 1996]. In this study a dual infection with GBV-C was detected in 8 (31%) of 26 HCV infected patients. All these patients were positive both by PCR and by a second-generation anti-HCV EIA. The lower age of the hemophiliacs with GBV-C infections as compared to those with HCV infections indicate that the risk for this group of patients to acquire GBV-C infection is higher than that for HCV infection. There was a high frequency of infections due to both GBV-C and HCV in the present study, considering the rather low prevalence of HCV, 0.5%, observed in donors from Nicaragua [Garcia et al., 1996].

Although the epidemiology of GBV-C remains to be clarified, recent studies in the United States and Japan have shown a relatively high frequency of GBV-C infections among blood donors, 1.7% and 0.8%, respectively [Dawson et al., 1996; Linnen et al., 1996; Masuko et al., 1996]. Transmission of the virus has been demonstrated to occur by blood transfusions and by other parenteral modes [Aikawa et al., 1996; Masuko et al., 1996; Schmidt et al., 1996] as well as vertically from mother to newborn child [Feucht et al., 1996]. The virus has been found in patients suffering from both acute and chronic cryptogenic hepatitis [Simons et al., 1995; Fiordalisi et al., 1996; Leary et al., 1996] and has also been implicated in fulminant hepatitis [Yoshiba et al., 1995; Heringlake et al., 1996], although this outcome of GBV-C infection still remains controversial [Kuroki et al., 1996; Kao et al., 1996].

The finding of genotype 1a of HCV in Nicaragua was anticipated, since this genotype is prevalent in the Americas [Andonov and Chaudhary, 1994; Cha et al., 1992; Simmonds et al., 1993; Stuyver et al., 1993] but is also distributed worldwide [McOmish et al., 1994]. Some HCV genotypes (i.e., 4–11) are mainly confined to restricted areas in Africa and Asia [Cha et al., 1992; Simmonds et al., 1994; Mellor et al., 1995; Tokita et al., 1994a, 1994b, 1995]. However, none of these genotypes was found in the studied population. Therefore, GBV-C strains sharing sequence similarities to strains with Asian background was unexpected. A possible explanation for this finding would be that the GBV-C strains in Central America have their origins in Amerindian populations, since all the hemophiliacs had been treated only with locally derived blood products and no commercial cryoprecipitates from the United States or Japan had been used. Molecular epidemiology may, apart from its use in tracing routes of viral transmission as for HBV [Magnius & Norder, 1995], HCV [Chou et al., 1993; Davidson et al., 1995], and GBV-C [Egawa et al., 1996; Schmidt et al., 1996], also be used as a tool to study prehistoric and historic migrations of human population as for, e.g., HTLV I [Yanagihara, 1994; Song et al., 1995] and HBV [Norder et al., 1993]. Recent phylogenetic studies on HTLV II sequences, as well as genetic studies, favour the idea that the ancestral migration waves preceeding the settlement of the New World have origins in Mongolia, Manchuria, or Southeast Siberia [Hall et al., 1994; Neel et al., 1994; Biggar et al., 1996]. That Amerindian viral strains may be prevalent in Central American populations with Hispanic background was recently described for HBV, where the prevailing genotype F strains also are the strains prevailing in the Amerindian populations of the Americas [Arauz-Ruíz et al., 1997]. The similarity of Nicaraguan GBV-C strains to strains from Asia, suggesting that these strains have Amerindian origin, indicates that GBV-C is an extremely conserved virus and explains why the divergence of GBV-C strains with different geographical origin is much lower than that for HCV. Future studies will establish whether the geographical variants of GBV-C, although most likely representing ancient splittings, diverge to the extent needed to define them as genotypes of GBV-C.

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